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Phagocytic uptake of polystyrene microspheres by alveolar macrophages: effects of the size and surface properties of the microspheres

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Abstract

Polystyrene microspheres with diameters of 0.2, 0.5, 1.0, 6.0 and 10 μm were added to alveolar macrophages, and their uptake was determined as the amount of superoxide generated from macrophages by the usage of chemiluminescence assay with luminol. The amount of superoxide generated was apparently higher with polystyrene microspheres with a diameter of 1 μm than those with diameters smaller than 1 μm (i.e. 0.2 or 0.5 μm) and with larger than 1 μm (6 or 10 μm). The effects of the functional groups located on the microsphere surfaces upon the uptake by alveolar macrophages were studied with polystyrene microspheres of 1 μm diameter having the primary amine, sulfate, hydroxyl, or carboxyl groups on their surfaces. We found that the macrophages most effectively trapped polystyrene microspheres with primary amine groups, those with carboxyl groups to a slightly lesser extent, and other microspheres much less amounts. The surface properties of these microspheres were determined by measuring their electrophoretic mobility in phosphate buffer solution (pH 7.4) with various ionic strengths. By the analysis of data with Ohshima's electrokinetic theory for soft particles, the surface charge density and the electrophoretic softness of the microsphere surfaces were determined. All the microsphere surfaces were found to be negatively charged, and those with primary amine groups and carboxyl groups were softer than other microspheres. From these findings, it is suggested that microspheres having soft surfaces are easily accessible to alveolar macrophages, and effectively trapped by macrophages.
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Keywords: Alveolar macrophage; Phagocytic; Size; Charge; Softness

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1. Introduction

Recently, we have reported that polystyrene microspheres with an average diameter of 1 μm are easily recognized by macrophage-like differentiated HL60 [1]. Polystyrene microspheres with diameters less than 0.2 μm and more than 10 μm were not engulfed by HL60. Also, polystyrene microspheres having primary amine groups on their surfaces were most effectively engulfed by the macrophages, as compared with those having sulfate, hydroxyl and carboxyl groups. When drug carriers, such as liposomes, microspheres, and nanospheres, are intravenously injected, they are rapidly trapped by cells of the mononuclear phagocyte system (MPS). This is the major limitation for drug targeting in general [2].

On the other hand, macrophages act as reservoirs for viruses such as HIV, playing a detrimental role during the progress of AIDS [3,4]. In the case of tuberculosis, tubercle bacillus coming from the air stays stable in alveolar macrophages for a long time [5,6]. Also, the lung is highly advantageous as a non-parenteral rout of drug delivery, and pulmonary delivery system has been thought to be effective drug delivery system for various compounds especially peptides and proteins [7–9].

This paper deals with the effects of size and surface properties of microspheres upon the phagocytic uptake of the microspheres by alveolar macrophages.

2. Experimental

2.1. Materials

Nemubutal injection (50 mg ml^{-1}) was purchased form Dainippon Pharmaceutical, Japan. Phenolred free eagle MEM (EMEM) medium and phosphate buffer solution (PBS without containing Ca^{2+} and Mg^{2+}) were from Nissui Pharmaceutical, Japan. Luminol was purchased from Tokyo Kasei Organic Chemicals, Japan. Each kind of polystyrene microsphere suspension (Polybeads™ Microspheres), polystyrene microspheres and polystyrene microspheres having primary amine, sulfate, hydroxyl, or carboxyl groups on their surfaces, was purchased from Polysciences, USA. Activated carbon (SHIRASAGI™ LPK-424-2) was a gift from Takeda Chemical Industries, Ltd. All other chemicals were of reagent grade.

2.2. Cell isolation

Alveolar macrophages were recovered from 5-week-old female F344/NSlc rats (70–90 g) by broncho alveolar lavege (BAL). Rats were anethsized by an intraperitoneal injection of nembutal (150 mg kg^{-1} body weight). Subsequently, a cross-section exposing the underlying intestines were made, and the rat was completely exsanguinated by serving the abdominal aorta. The trachea was exposed and a Elaster (Hakko Medical, Japan) was used for tracheal cannulation. The lung was lavaged using intratracheal instillation of 3 ml calcium magnesium free Dulbecco's balanced phosphate buffer (PBS^-) eight times, followed by the intratracheal instillation of 5 ml PBS^- for four times at 37 °C. Macrophages isolated from lung lavage fluid by centrifugation at 800 rpm for 8 min were resuspended in EMEM medium containing 10% FCS. Aliquots of 1 ml of 10% PCS in EMEM containing 5×10^5 cells were placed in a container for a Luminometer (UPD-400, Meidensha, Japan) and incubated in a humidified chamber at 37 °C with 5% CO_2 overnight. After 1 night incubation, the media containing non-adherent cells were withdrawn. Subsequently, aliquots of 1 ml of EMEM containing 2.24 mm L-glutamine and 100 IU ml^{-1} penicillin–streptomycin) were added, and then it was kept in a humidified chamber at 37 °C with 5% CO_2 for 30 min.

2.3. Superoxide anion assays

The amount of superoxide produced from macrophages by the addition of polystyrene microspheres was determined by chemiluminescence (CL) assay using a Luminometer (UPD-400, Meidensha, Japan). Luminol was dissolved in dimethyl sulfoxide (DMSO) to be a final concentration of 10 mg ml^{-1} (56 mM). Macrophages

were cultured in a Luminometer container, then the luminol solution (20 μl) was added to 1 ml of macrophage culture system to be the final concentration of 1×10^{-3} M, and 15 min later the polystyrene microspheres were added.

2.4. Measurements of the electrophoretic mobility of microspheres

The electrophoretic mobility of polystyrene microspheres was measured in Dulbecco's buffer solution at pH 7.4 with various ionic strengths by using an automated electrokinetic analyzer (Zetasizer, Malvern, UK) at 37 °C. The measurement was repeated at least five times. The ionic strength was adjusted by dilution of Dulbecco's buffer solutions with an ionic strength of 154 mM with distilled water.

3. Results and discussion

3.1. Uptake of activated carbon by macrophages

Microscopic observation showed that the majority of alveolar macrophages started to uptake activated carbon 15 min after their addition to the culture medium (Fig. 1).

3.2. Effects of particle sizes of polystyrene microspheres

The uptake of polystyrene microspheres having various diameters (0.2, 0.5, 1.0, 6.0, and 10.0 μm) by alveolar macrophages was studied. Luminol solution was added to the culture system 15 min. prior to the addition of polystyrene microspheres. CL counts for 30 s due to phagocytosis-related superoxide generation was measured 2 h after the addition of polystyrene microspheres to macrophages. As clearly observed in Fig. 2, when polystyrene microspheres with diameters of 10 and 0.2 μm were added to the macrophage system, their CL intensities were almost the same as that of control (PBS^- solution), showing that the microspheres were not engulfed by macrophages. In contrast, the CL intensities were slightly greater for polystyrene microspheres with diameters between 0.5 and 6 μm, and that for 1 μm microspheres was greatest. The microspheres having diameters of 1 μm were most effectively trapped by macrophages, showing the highest CL intensity at any time after the addition of the. These results were supported by microscopic observation, in which alveolar macrophages engulfed effectively polystyrene micro-spheres with 1 μm diameter 2 h after the addition of the microspheres (Fig. 3).

3.3. Effects of surface properties of microspheres

In order to know the effects of the surface properties of micro-spheres upon the uptake of macrophages, generation of superoxide was serially monitored by addition of polystyrene microspheres of 1.0 μm diameter having various groups on their surfaces, such as primary amine, sulfate, hydroxyl, or carboxyl groups. As shown in Fig. 4, the CL intensity increased in the initial 15 min on addition of microspheres, then it decreased in the next 15 min attaining a steady level. The extent of phagocytic activity was evaluated from the CL intensity in the steady level between 30 min and 120 min [10].

The CL intensity with polystyrene microspheres having primary amine groups was highest and that with carboxyl groups was slightly lower than that with primary amine groups, both being much higher than those containing other functional groups. It is noteworthy that the intensity of microspheres without any functional group (the non-modified polystyrene microspheres) was very low. These results showed that alveolar macrophages trapped polystyrene microspheres with primary amine and carboxyl groups very effectively, those with other functional groups to certain extents and the non-modified polystyrene microspheres only a little, being essentially consistent with the results with macrophage-like differentiated HL60 cells, as reported before [1].

3.4. Surface properties of polystyrene microspheres

Information about the surface properties of polystyrene microspheres has been obtained from the electrophoretic mobility measurement of the microspheres dispersed in electrolyte solutions

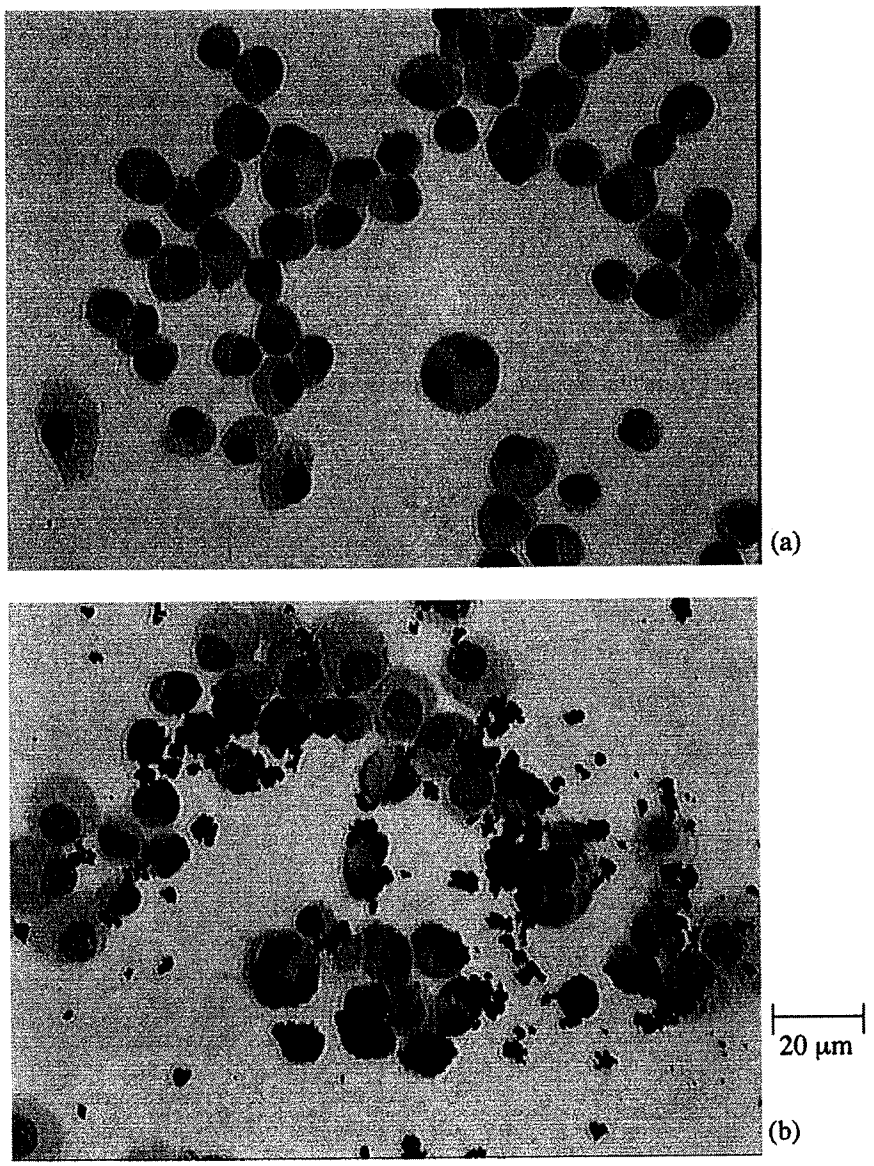

Fig. 1. Microscopic observations of alveolar macrophages (a) and those after the addition of activated carbon (b). The alveolar macrophages were fixed with methanol and then stained with Giemsa's solution.

with various ionic strengths. Hence, the electrophoretic mobilities of polystyrene microspheres having various functional groups on the surfaces were determined in terms of ionic strength of the dispersing medium at 37 °C, as shown in Fig. 5. The obtained mobilities with all the microspheres took negative values at all ionic strengths at pH 7.4, implying that their surfaces have net negative charges, although styrene itself does not carry the negative charge. Possibly, the negative charge was originated from polymerization of styrene monomer with an initiator.

With increase in the ionic strength, the negative electrophoretic mobility was decreased with all microspheres. The electrophoretic mobility, however, tended to become a non-zero value even in

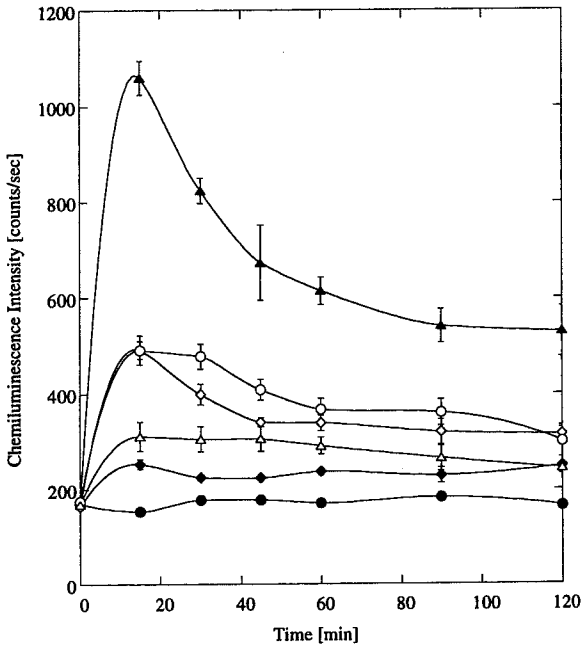

Fig. 2. Effects of sizes of polystyrene microspheres on the superoxide release from alveolar macrophages. Polystyrene microspheres with diameters of ◆, 0.2; ◇, 0.5; ▲, 1.0; ○, 6.0; △, 10.0 μm and ●, PBS^- (control) were added to macrophages. The data are taken from three experiments, each carried out in triplicate. Bars indicate the standard error of the mean (S.E.M.).

the solution with the ionic strength of as high as 0.154 M suggesting that the surface of the particles are 'soft' and results can be discussed in terms of the Ohshima's electrokinetic theory for 'soft' particles covered by ion-penetrable surface layers [11].

In this model, it is supposed that the particle having ionized groups of valency z on the surface uniformly distributed at a number density of N (m^{-3}) moves in a liquid containing a symmetrical electrolyte of valency v in the applied electric field, (n (m^{-3}) is the bulk concentration of symmetrical electrolytes in the dispersing medium.) The electrophoretic mobility μ is then expressed by Eq. (1) [11].

$$\mu = \frac{\varepsilon_r \varepsilon_o}{\eta} \frac{\psi_o/\kappa_m + \psi_{DON}/\lambda}{1/\kappa_m + 1/\lambda} f\left(\frac{d}{a}\right) + \frac{zeN}{\eta\lambda^2} \quad (1)$$

with

$$f\frac{d}{a} = \frac{2}{3}\left[1 + \frac{1}{2(1 + d/a)^3}\right] \quad (2)$$

$$\psi_{DON} = \frac{kT}{ve} \ln\left[\frac{zN}{2vn} + \left\{\left(\frac{zN}{2vn}\right)^2 + 1\right\}^{1/2}\right] \quad (3)$$

$$\psi_o = \frac{kT}{ve}\left(\ln\left[\frac{zN}{2vn} + \left\{\left(\frac{zN}{2vn}\right)^2 + 1\right\}^{1/2}\right] + \frac{2vn}{zN}\left[1 - \left\{\left(\frac{zN}{2vn}\right)^2 + 1\right\}^{1/2}\right]\right) \quad (4)$$

$$\lambda = \left(\frac{\gamma}{\eta}\right)^{1/2} \quad (5)$$

$$\kappa_m = \kappa\left[1 + \left(\frac{zN}{2vn}\right)^2\right]^{1/4} \quad (6)$$

$$\kappa = \left(\frac{2ne^2v^2}{\varepsilon_r\varepsilon_o kT}\right)^{1/2} \quad (7)$$

Here, a is the particle radius, d the thickness of the ion-penetrable surface layer, η the viscosity, γ the frictional coefficient of the surface layer, ε_r the relative permittivity of the solution, ε_0 the permittivity of a vacuum, ψ_{DON} the Donnan potential of the surface layer, ψ_0 the potential at the boundary between the surface layer and the surrounding solution, and κ is the Debye–Hückel parameter. We call ψ_0 the surface potential of a 'soft' particle and κ_m can be interpreted as the Debye–Hückel parameter in the surface layer. The parameter λ

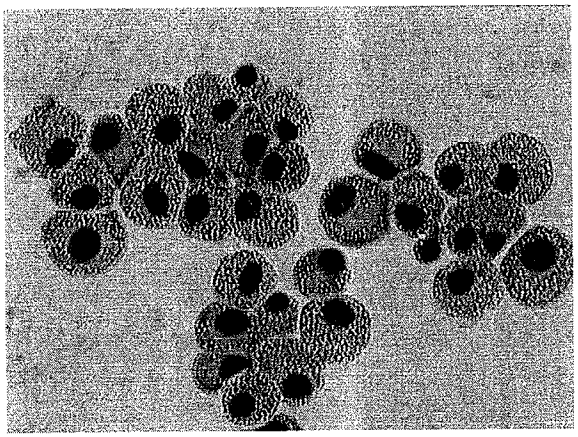

Fig. 3. Microscopic observation of alveolar macrophages 2 h after the addition of polystyrene microspheres with diameters of 1 μm. The alveolar macrophages were fixed with methanol and then stained with Giemsa's solution.

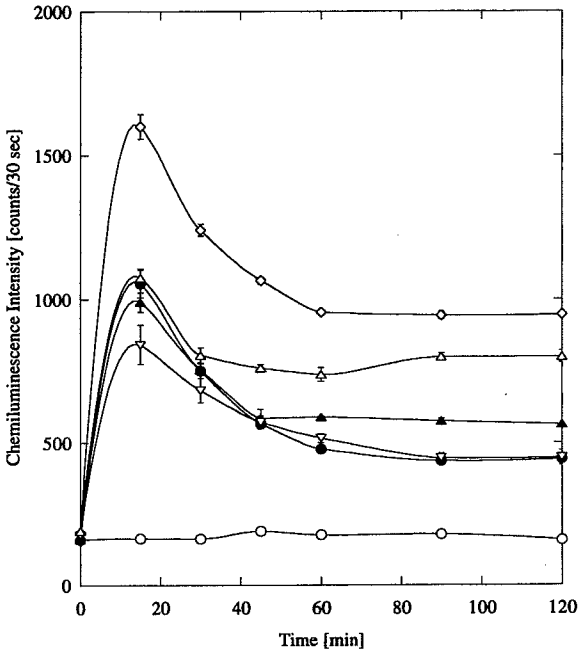

Fig. 4. Effects of functional groups existing on the polystyrene microsphere surfaces on the superoxide release from alveolar macrophages. Polystyrene microspheres having ◇, primary amine; ▽, sulfate; ▲, hydroxyl; △, carboxyl groups on their surfaces; ●, unmodified polystyrene microspheres, and ○, PBS^- (control) were added to macrophages. The data are taken from three experiments, each carried out in triplicate. Bars indicate the S.E.M.

characterizes the degree of friction exerted on the liquid flow in the surface layer and zN represents the number density of the fixed charges in the surface layer.

The reciprocal of λ ($1/\lambda$) has the dimension of length and can be considered to be an electrophoretic 'softness' parameter, since in the limit $(1/\lambda) \rightarrow 0$, the surface layer becomes rigid. Eq. (1) involves two unknown parameters, N and $1/\lambda$, which now represent the fixed charge density in the microsphere and its softness, respectively. By a curve-fitting procedure reported previously [12,13], values of zN and $1/\lambda$ were determined.

The value of μ calculated via Eq. (1) was plotted against the ionic strength in comparison with the experimental data in Fig. 5 (solid lines). It is possible to draw a curve with a pair of single values of each of zN and $1/\lambda$, resulting in good agreement with the experimental data over a wide range of ionic strength. Namely, the microsphere surface can be considered as a 'soft surface' described by Eq. (1). Thus this agreement enables us to estimate the values of the unknown parameters zN and $1/\lambda$, by a curve-fitting procedure. In the calculation, we used the values of the relative permittivity ε_r and the viscosity η of water. The best-fit values of the charge density (zN) and the softness parameter ($1/\lambda$) of the surfaces of polystyrene microspheres with various functional groups on their surfaces are summarized in Table 1.

As clearly observed in Table 1, much smaller values of the softness parameter ($1/\lambda$) are obtained in the surface layers of the polystyrene microspheres having primary amine groups and carboxyl groups than those having other functional groups, showing that the surfaces of the polystyr-

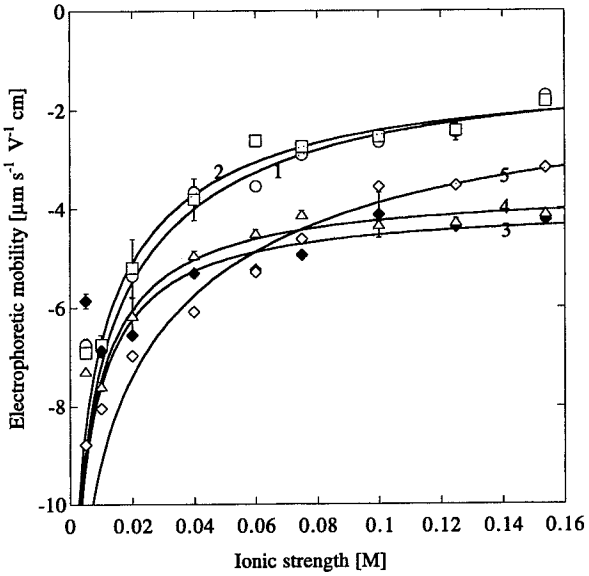

Fig. 5. Electrophoretic mobility of polystyrene microspheres having various functional groups on the surfaces. Symbols are the experimental data with polystyrene microspheres having ◆, primary amine; □, sulfate; ◇, hydroxyl; △, carboxyl groups on their surfaces; and ○, unmodified polystyrene microspheres were dispersed in pH 7.4 buffer solutions. The data are taken from three experiments, each carried out five times. Bars indicate the S.E.M. Solid lines are theoretical ones calculated with $zN = -0.177$ M, and $1/\lambda = 0.626$ nm (curve 1); $zN = -0.131$ M, and $1/\lambda = 0.812$ nm (curve 2); $zN = -0.051$ M, and $1/\lambda = 2.370$ nm (curve 3); $zN = -0.056$ M, and $1/\lambda = 2.164$ nm (curve 4); $zN = -0.310$ M, and $1/\lambda = 0.578$ nm (curve 5).

Table 1
The values of zN and $1/\lambda$ of various kinds of polystyrene microspheres

Types of polystyrene microspheres	zN (M)	$1/\lambda$ (nm)
Intact polystyrene microspheres	−0.177	0.626
Polystyrene microspheres with primary amine groups	−0.051	2.370
Polystyrene microspheres with carboxyl groups	−0.056	2.164
Polystyrene microspheres with hydroxyl groups	−0.310	0.578
Polystyrene microspheres with sulfate groups	−0.131	0.812

ene microspheres having primary amine groups and carboxyl groups are much softer than the surfaces of other microspheres. The values of surface charge density of all kinds of microspheres are negative, while the values of the microspheres having primary amine groups and carboxyl groups are less negative than those having other functional groups. Also, the surface charge of the alveolar macrophages is considered to be negative, like usual biological cells [12,13]. Therefore, it is suggested that microspheres having soft surfaces are easily accessible to alveolar macrophages. When microspheres with soft surfaces come close to alveolar macrophages, macrophages may detect the primary amine groups on the microsphere surfaces. Then the microspheres having primary amine groups are considered to be most effectively engulfed by macrophages.

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